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PRINCIPAL INVESTIGATOR: Lurong Zhang, M.D., Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
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6. AUTHOR(S)
Lurong Zhang, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

e-mail:
zhangl@gusun.georgetown.edu

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13. ABSTRACT ***(Maximum 200 Words)***

The tumor-stroma interaction plays critical role in breast cancer progression. We hypothesize that hyaluronidase (HAase) produced by malignant tumor cells can sever as switch to release growth factor that immobilized in extracellular matrix (ECM), and then enhance the tumor progression. We have demonstrated: (1) exogenous HAase stimulated the colony formation via shift the FGF-2 from immobilized form to free form; (2) transfection of HAase cDNA into FGF-2 high expressing SW13 cells could stimulate colony formation; and (3) transfection HAase cDNA into MDA231 cells could enhance the tumor growth in CAM system. In past year, we further characterized the functions of HAase and performed the underlying mechanism studies in a great detail. The HAase expression by transfectants was confirmed by Western blot and the HAase activity was determined by ELISA like assay. The HAase released FGF-2 could be detected by Western blot. The bioactivity of FGF-2 was demonstrated by stimulation of phosphorylation of tyrosine and MAPK, which in turn, enhanced the anchorage-dependent and anchorage-independent growth of MDA231-HAase transfectants. Furthermore, the conditioned media of transfectants could stimulate the growth of endothelial cells. The in vivo studies indicated that the HAase transfectants formed aggressive tumor via enhancement of angiogenesis. However, we did not see the increased metastases. We have obtained antibody against HAase by immunization of rabbit with synthetic peptide. The antibody has been further purified with affinity column and will be utilized for determination of the level of HAase expression in breast cancer samples.
This study further proved that HAase play a role in tumor progression and may be a target for tumor therapy.

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INTRODUCTION

The tumor-stroma interaction plays critical role in breast cancer progression (1-15). Based on observations made by other researches and us (16-36), we hypothesize that hyaluronidase (HAase) produced by malignant tumor cells can sever as a switch to release growth factor immobilized in extracellular matrix (ECM), and then enhance the tumor progression.

In last year, we have demonstrated: 1) exogenous HAase stimulated the colony formation via shift the FGF-2 from immobilized form to free form; 2) transfection of HAase cDNA into SW13, a FGF-2 high expressing cell line, could stimulate colony formation; and 3) transfection HAase cDNA into MDA231 cells could enhance the tumor growth in CAM system.

In this year, we further characterized the MDA 231 cells transfected with PH-20 (cDNA cording for human membrane-bound HAase) and performed the underlying mechanism studies in a great detail. The HAase expressed by transfectants was confirmed by Western blot. The HAase activity in the lysate of transfectants was determined by ELISA like assay. The HAase released FGF-2 was detected by Western blot. The bioactivity of FGF-2 was demonstrated by stimulation of phosphorylation of tyrosine and MAPK, which in turn, enhanced the anchorage-dependent and anchorage-independent growth of MDA231-PH-20 transfectants. Furthermore, the conditioned media of transfectants could stimulate the growth of endothelial cells. The *in vivo* studies indicated that the HAase transfectants formed aggressive tumors via enhancement of angiogenesis. However, we did not see the increased lung metastases.

We have obtained antibody against HAase by immunization of rabbit with synthetic peptide. The antibody has been further purified with affinity column and will be utilized for determination of the level of HAase expression in breast cancer samples.

This study further proved that HAase plays a role in tumor progression via release of FGF-2 and stimulation of growth of tumor and endothelial cells, which may be a target for tumor therapy.

BODY

Aim 1. To test the hypothesis that over-expression of HAase increases the malignant potential of tumor cells.

In 1999, we had used two approaches to obtain a high level of HAase in tumor cells. One is to directly add exogenous HAase to the tumor cells, and the other is to transfect the cDNA of HAase into tumor cells.

In 2000, we have concentrated our study on HAase transfected MDA 231 breast cancer cells, since the gene transfection is the best way to identify the function of a given molecule, simply due to the same background between the vector alone and interesting gene transfectants.

To confirm the success of the transfection of MDA 231 breast cancer cells with PH-20 cDNA, a Western blotting was performed using the antibody generated against bovine testicular HAase in 1999 when this project was started. The result (**Fig 1**) showed that while there was no band can be detected with anti-HAase in the cell lysate of vector alone transfectants, there was a strong band appeared in the PH-20 transfectants.

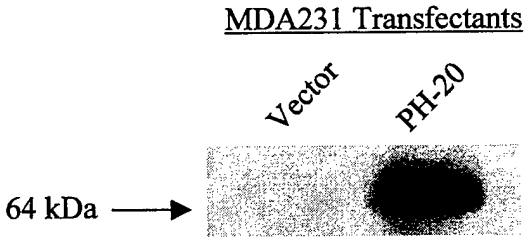

Fig 1. Detection of PH-20 expression in MDA231 transfectants by Western bolt. Thirty μg of cell lysate was electrophoreszed on 8% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with rabbit anti-bovine testicular HAase (1:1000), HRP-anti-rabbit (1:4000) followed by ECL.

To see if this immuno-reactive PH-20 does have its bioactivity, a HAase ELISA-like assay was conducted. With the increased addition of lysate from PH-20 transfectants, an increased amount of coated HA was digested and the less of bound HA could be detected by biotinylated HA binding (**Fig 2**). This indicates that the HAase produced by PH-20 transfectants possesses the ability to digest HA.

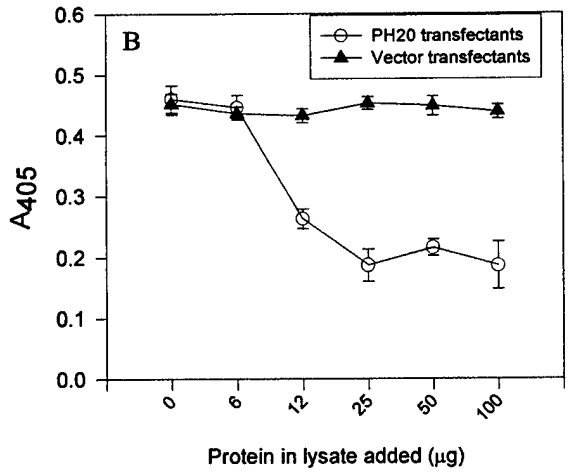

Fig 2. ELISA for activity of PH-20 in cell lysate of MDA231 transfectants. Different amounts of cell lysate were added to the HA coated ELISA plate and incubated at 37°C overnight. The digested HA were washed away, and the HA remained in the plate was detected by biotinylated HA binding protein followed by HRP streptavidin and substrate.

In previous study, we demonstrated that when exogenous HAase was added to culture media of SW13 and MDA468 cells, the FGF-2 was released from the immobilized portion to the free media portion. To see if this holds-up in cells expressing high level of HAase, the conditioned media from PH-20 transfectants was

examined for FGF-2 by enrichment with heparin-affinity column followed by Western blotting. The result (**Fig 3**) showed that FGF-2 was significantly increased in the media of PH-20 transfectants compared to the control, indicating that endogenously expressed HAase indeed can shift the immobilized FGF-2 to the free portion, which is required for its activity.

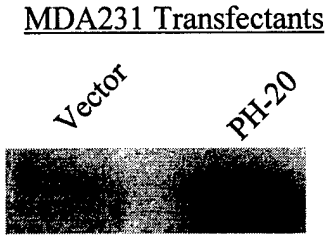

Fig 3. FGF2 was released by PH-20 in conditioned media of MDA231 transfectants. Six ml of conditioned media harvested from some density of MDA231 transfectants was mixed with heparin-Sepharose 4B at 4°C overnight. After washed with 0.3 M NaCl, 30 µl of loading buffer were added to the beads, boiled and electrophoresed on 8% SDS-PAGE. The Western bolt was carried out with anti-FGF2 (1:1000).

We then wanted to see if the released FGF-2 could exert its biofunction. Four isoforms of FGF receptors are expressed by MDA231 cells and can tranduce the signal via tyrosine kinase and MAPK pathway. The lysate from the transfectants were examined for phosphorylation of tyrosine and MAPK. The results of Western blotting (**Fig 4**) showed that the phosphorylated tyrosine and MAPK were increased in the PH-20 transfectants, suggesting that the HAase released FGF-2 could trigger the MAPK signal pathway via the paracrine effect.

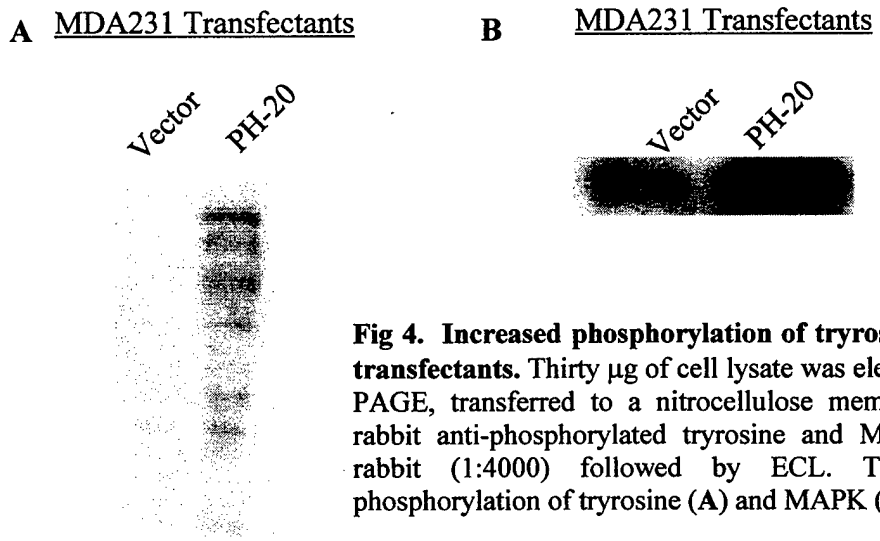

Fig 4. Increased phosphorylation of tryrosine and MAPK in PH-20 transfectants. Thirty µg of cell lysate was electrophoreszed on 8% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with rabbit anti-phosphorylated tryrosine and MAPK (1:1000), HRP-anti-rabbit (1:4000) followed by ECL. There was an increased phosphorylation of tyrosine (**A**) and MAPK (**B**) in PH-20 transfectants.

If the paracrine effect is true in the HAase high expression condition, then the cell growth is likely to be stimulated. To check this out, an anchorage-dependent growth assay was performed. The result (**Fig 5**) showed when cultured in the 24 well plate, the PH-20 transfectants grew faster than the vector alone transfectants, especially in later stage of culture (day 4 and 5), indicating that there may be a accumulation effect of both HAase and FGF-2.

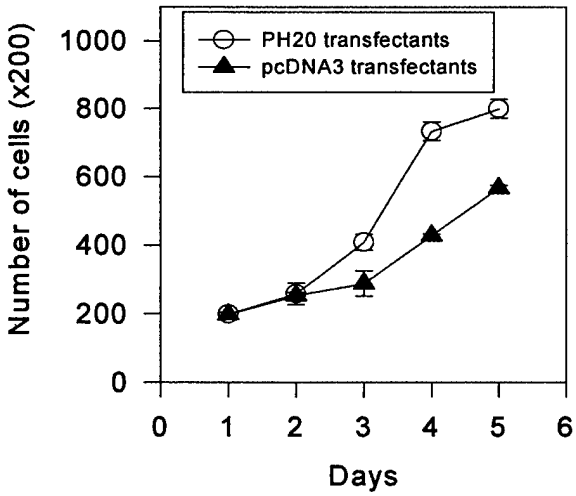

Fig 5. Effect of PH-20 on anc dependent growth of transfectants. T were seeded in 24 well plate, harve indicated day and counted with counter. The PH-20 stimulated the gr transfected cells compared to vector ($P<0.05$).

Colony formation is one of the characteristics of malignancy tumor cells. To see if this is enhanced, a colony formation assay was conducted in soft agar. The PH-20 cells formed much bigger colony than vector alone control cells (**Fig 6 A**) and the number of colonies bigger than 60 μm in diameter was double in PH-20 cells compared to the control (**Fig 6 B**). Again, it is proved that HAase released FGF-2 could enhance the anchorage-independent growth of PH-20 cells.

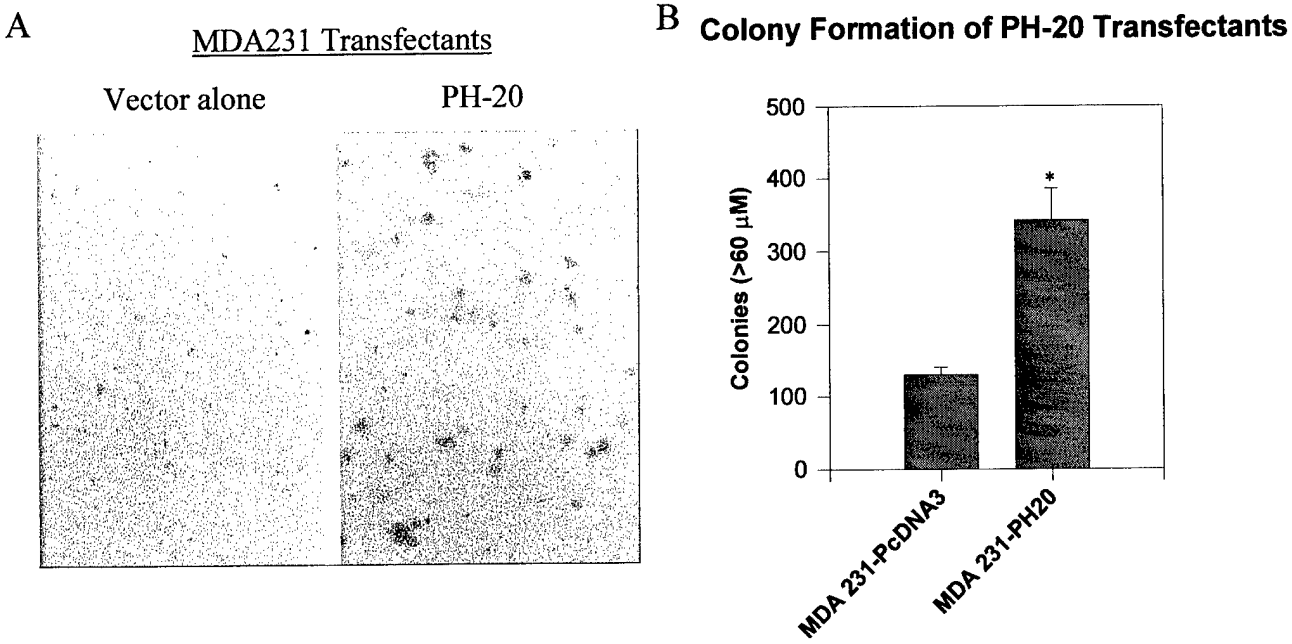

Fig 6. Effect of PH-20 on anchorage-dependent growth of transfectants. Twenty thousand transfected be mixed with 0.18% of agarose in 10% FBS-DMEM and then placed on top of 0.36% agarose. Two weeks later, the colonies (>60 μm) were pictured (**A**) and quantified (**B**) using an Omnicon Image system. PH-20 transfectants formed much more big colonies than mock controls (P<0.01).

It has been known that FGF-2 is a mitogen for both tumor cells and endothelial cells. We speculate that the HAase released FGF-2 may also act on endothelial cells. To test this, the conditioned media (CM) from transfectants was added to media of endothelial cells, and the cell number was counted on day 4. The result (**Fig 7**) showed that the growth of endothelial cells was greatly stimulated by CM of PH-20 cells in a dose-dependent manner compared to the control. This dual effect of HAase will enhance its promoting role in tumor progression via stimulation of both tumor cells and endothelial cells.

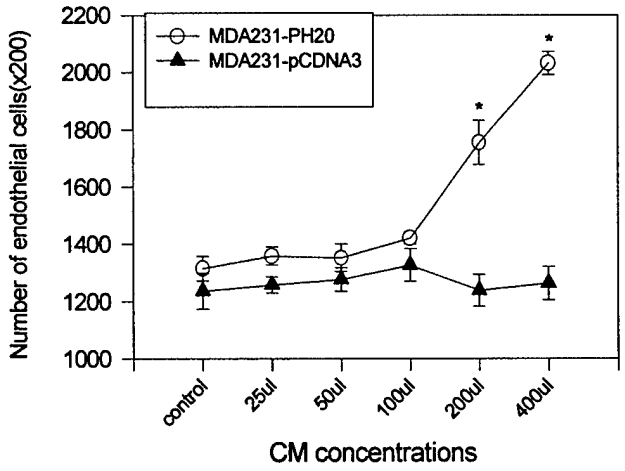

Fig 7. Effect of CM on growth of endothelial cells. The different amount of conditioned media harvested from similar density of cells was added to adult bovine aortic endothelial (ABAE) cells. The cells were harvested on day 4 and counted. The CM from PH-20 transfectants stimulated the growth of endothelium compared to the controls (P<0.01).

To see the HAase enhanced malignant phenotype *in vitro* can be translated *in vivo*, the tumor growth on CAM and in nude mice model was examined. When one million of PH-20 cells were placed on the top of 10 days chicken embryo, they formed bigger tumors than vector control cells (**Fig 8**).

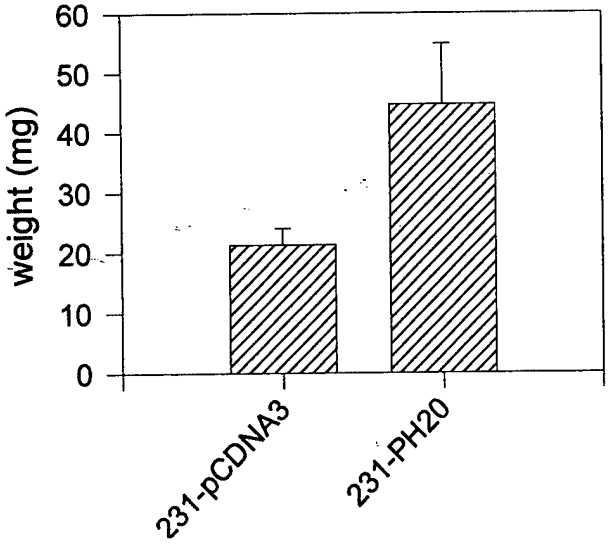

Fig 8. Effect of PH-20 on tumor growth on CAM. The vector or PH-20 transfectants (1 x 10^6) were placed on of CAM of 10 days old chicken embryo (15eggs/group) and four days later, the tumors formed on CAM photographed (**A**), harvested and weighted (**B**). The PH-20 transfectants formed bigger tumor than vector transfect

This was further confirmed by nude mice model. Two millions of MDA 231 cells transfected with either vector alone or PH-20 were subcutaneously injected into 5 weeks-old nude mice (5 /group). Four weeks later, the mice were photographed, and the tumors were harvested and weighted. The results (**Fig 9**) showed that the tumors formed by PH-20 transfectants were much bigger than that formed by control vector transfectants, indicating that the HAase could enhance the tumor growth *in vivo*.

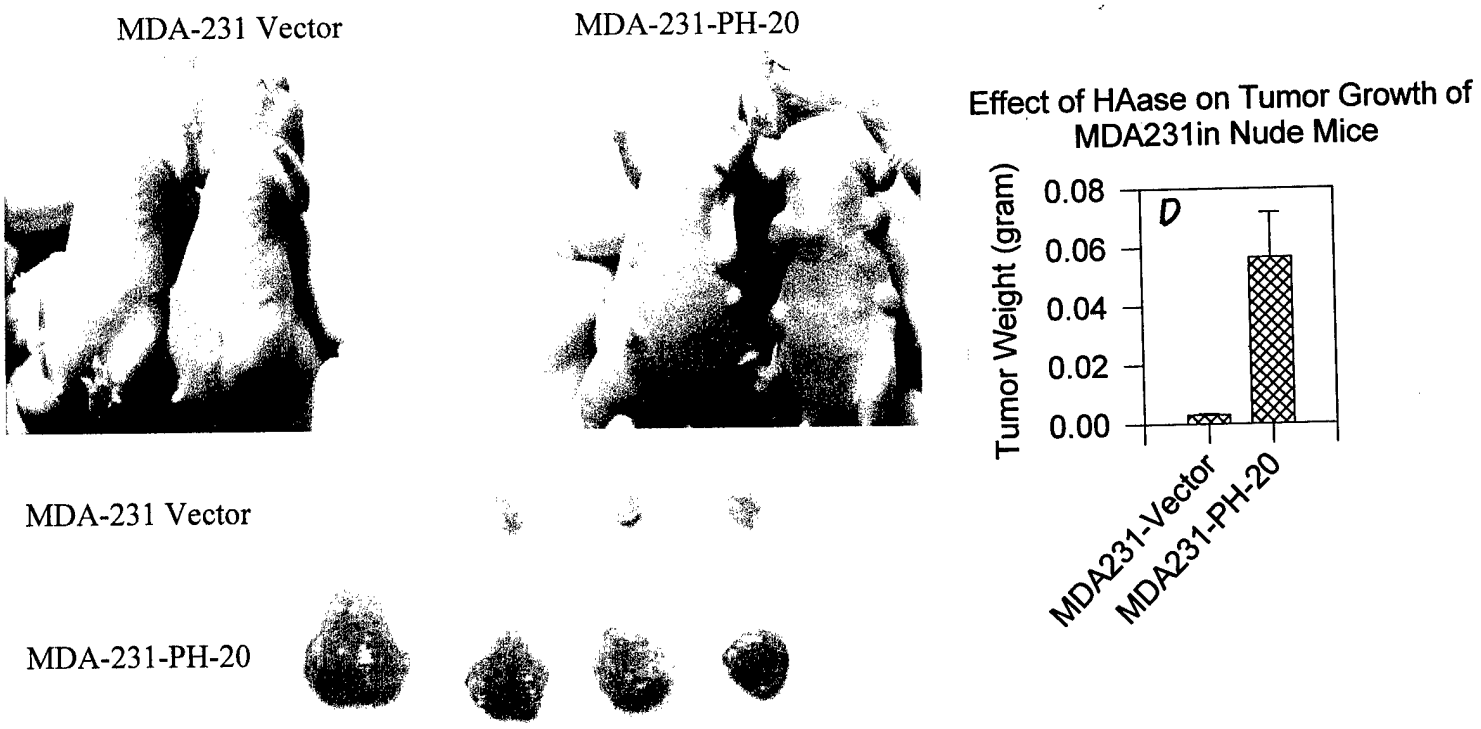

Fig 9. Effect of PH-20 on tumor growth in nude mice. Two millions of MDA 231 cells transfected with either vector alone or PH-20 were subcutaneously injected into 5 weeks-old nude mice (5 /group). Four weeks after inoculation, the mice were photographed (**A** and **B**), and the tumors were harvested (**C**) and weighted (**D**). The PH-20 cells formed bigger tumors than the vector alone control.

However, when the mice lung were carefully examined, we did not find that there was an increased metastases. This may due to the "wrong seeds" or "wrong soil". The tumor metastasis is a complex process, which requires not only the enzymes to digest the ECM and basement membrane of vessels, but also the adhesion molecules to settle down. HAase alone is not enough for induction of metastasis in the cells that lack of other necessary molecules.

Since FGF-2 is a potent angiogenesis factor and can be released by HAase, we speculated that the tumors formed by PH-20 transfectants might contain more newly formed vessels than the control tumors. To test this, the vessels in the tumors were examined by immunohistochemic staining with antibody against factor VIII expressed on endothelial cells. The results (**Fig 10**) showed that the vessels in the tumors formed by PH-20 transfectants were more than that in the control tumors, indicating the angiogenesis is enhanced by high expression of HAase. Besides of FGF-2, the small molecule weight HA digested by HAase may also contribute to this increased angiogenesis (31). Whether there is other angiogenic molecule involved needs to be investigated.

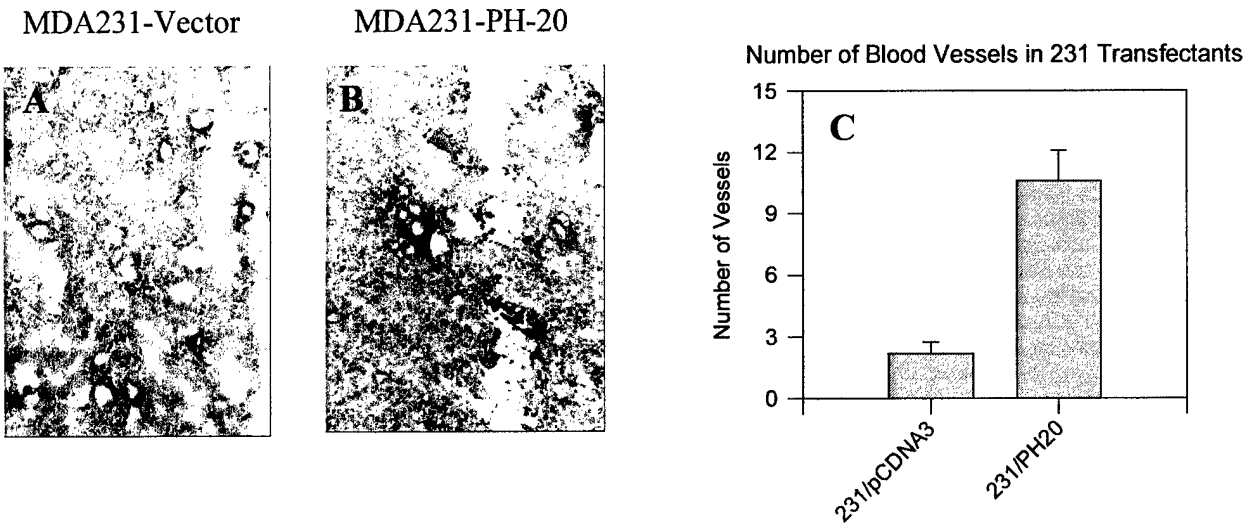

Fig 10. Effect of PH-20 on tumor angiogenesis. The frozen sections of tumors formed by vector or PH-20 transfectants were stained for vessels with anti-endothelial antibody followed by AEC substrate (in red, **A** and **B**). The numbers of vessels stained were counted from 10 random fields and the PH-20 group had more vessels than the vector group (**C**).

Aim 2. To study the significance of membrane-bound HAase in human breast cancer tissues.

In last year, we used computer program to find out the possible antigenic domain in the extracellular portion of PH-20. The result showed that the peptides formed by amino acids from 148 to 167 and from 180 to 200 on N-terminal extracellular portion of PH-20 are hydrophilic and likely to be served as antigens. Therefore, two peptides (EEWRPTWARNWKPKDVYKNR and LTEATEKAKQEFEKAGKDFL) were synthesized and then conjugated to KLH to make full antigens. Two rabbits were immunized with each peptide. The high titer (!:5,000 to 10,000 on ELISA) of polyclonal antibodies was obtained.

In this year, we used affinity column to purify the antibody and obtained in IgG form (Fig 11).

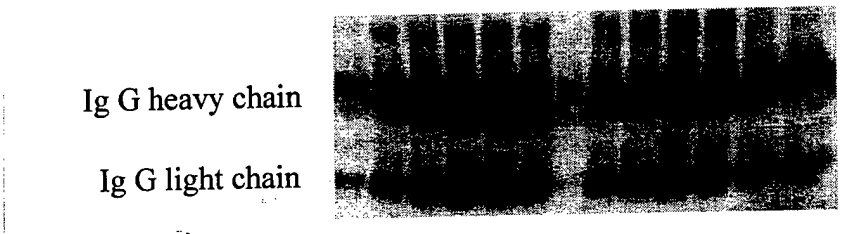

Fig 11. The affinity purification of anti-HAase. The rabbit serum containing antibodies against HAase peptide were diluted with PBS (1:1) and applied to protein A-Sepharose 4B column. After washed the unbound protein, the bound IgG fraction was eluted and electrophoresed on the 8% SDS-PAGE under reducing condition. The major bands are heavy chain and light chain, indicating there is few other protein contamination.

In next year, we will use the purified anti-HAase to stain the human breast cancer tissues to see if the expression of HAase is increased in the malignant tumor cells.

Key Research Accomplishments

- Characterized the expression of HAase in MDA231 transfectants and its bioactivity by Western blotting analysis and ELISA like assay.
- Detected HAase released FGF-2 by Western blot.
- Demonstrated that the HAase released FGF-2 could stimulate phosphorylation of tyrosine and MAPK.
- Demonstrated that anchorage-dependent and anchorage-independent growth of MDA231-HAase transfectants were enhanced.
- The conditioned media of transfectants could stimulate the growth of endothelial cells.
- The *in vivo* studies indicated that the HAase transfectants formed aggressive tumor via enhancement of angiogenesis. However, we did not see the increased metastases.
- Obtained antibody against HAase further purified with affinity column.

Reportable Outcomes

- **Manuscripts:**

1. Lurong Zhang, Zeqiu Han, Ivan Ding, Ningfei Liu, Weiming Liu, Jianzhong Xie, Feng Gao and Charles B. Underhill: Hyaluronidase acts as a switch for fobroblast growth factor.

2. Feng Gao, Ningfei Liu, Zeqiu Han, Charles B. Underhill and Lurong Zhang: Over-expression of human membrane-bound hyaluronidase (PH-20) promotes tumor growth.

- **Abstract and presentation:**

1. Lurong Zhang, Zeqiu Han and Charles B. Underhill: Hyaluronidase serves as a switch for basic fibroblast growth factor. Proc. Annu. Meet. Am. Assoc. Cancer Res 1999; 40: 460:3041

2. Feng Gao, Ningfei Liu, Zeqiu Han, Charles B. Underhill and Lurong Zhang: Over-expression of human membrane-bound hyaluronidase (PH-20) promotes tumor growth. Proc. Annu. Meet. Am. Assoc. Cancer Res 2000; 41: 133

- **Cell lines and antibody generated by supporting of this grant**

1. Human membrane-bound HAase high expressing MDA231 and SW13 cells.

2. Rabbit antibodies against human membrane-bound HAase.

CONCLUSIONS

1. To our knowledge, this study at first time reveals that HAase can sever as a switch to release FGF-2 from immobilized form to free active form and plays a role in tumor progression.

2. Our *in vitro* data with PH-20 transfected MDA 231 cells demonstrated that there is an increased phosphorylation of tyrosine and MAPK, and the enhanced anchorage-dependent and anchorage-independent growth in tumor cells expressing high level of HAase. This HAase released FGF-2 paracrine effect also exerted on endothelial cells.

3. Our *in vivo* data suggest that the tumor growth can be enhanced by expression of HAase via promoting of growth of tumor cells and angiogenesis.

4. The blocking of expression or activity of HAase may be a way to inhibit the tumor progression.

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Appendices

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#3041 Hyaluronidase acts as a switch for immobilized FGF2. Lurong Zhang, Zequi Han, Ivan Ding*, Jianzhong Xie*, Ningfei Liu and Charles B. Underhill. *Dept. of Cell Biology, Georgetown Univ. Med. Center, 3900 Reservoir Road, NW, Washington DC 20007; *Dept of Radiation Oncology, Univ. of Rochester School of Med and Dent., Rochester, NY.*

When many types of growth factors (GFs) are released by cancer cells, they are initially immobilized by the extracellular matrix (ECM) and can be active only after release. These GFs may be associated with the negatively-charged glycosaminoglycans, hyaluronan and chondroitin sulfate. Previous studies have shown that some types of malignant tumors express hyaluronidase (HAase), the enzyme that breaks down hyaluronan and chondroitin sulfate. Based on these observations, we hypothesized that HAase may induce the release of GFs associated with glycosaminoglycans and thereby stimulate tumor growth and angiogenesis. To test this possibility, we treated two human tumor cell lines, SW 13 and MDA 435, with testicular HAase, and found that in both cases, their ability to form colonies (>60 μm) in soft agar was increased by 4–5 fold compared to the control groups. The treatment with HAase also promoted the release of fibroblast growth factor 2 (FGF2) from the cell layer into the culture media, as detected by an ELISA. However, VEGF was not released by the treatment with HAase. These results suggest that the HAase-induced stimulation of tumor colony formation may be due to the release of FGF2 from the ECM. Interestingly, however, HAase did not stimulate the growth of these cells when they were directly attached to plastic substratum. This suggests that the effects of HAase are masked by the anchorage culture conditions through some unknown mechanism.

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#847 OVER-EXPRESSION OF HUMAN MEMBRANE-BOUND HYALURONIDASE (PH-20) PROMOTES TUMOR GROWTH. Feng Goa, Ningfei Lui, Zeqiu Han, Charles B Underhill, and Lurong Zhang, *Changzheng Hosp, Shanghai, P.R. China, and Lombardi Cancer Ctr, Georgetown Univ, Washington, DC*

Membrane-bound hyaluronidase (PH-20), that is normally only present on sperm, is expressed by some tumor cells, and by metastatic cells in particular. In previous studies, we have found that the addition of testicular hyaluronidase to cultured cells can release immobilized FGF2 and activate its biological activity. To further examine the function of PH-20 in tumor progression, we transfected the cDNA for PH-20 into MDA231, a human breast cancer cell line. The cells expressing PH-20 were confirmed by Western blotting and by an ELISA using hyaluronar (HA) as substrate. The PH-20 transfected cells were then characterized and found to have the following properties: 1) the colony formation ability was greatly enhanced; 2) medium conditioned by these cells stimulated the growth of cultured endothelial cells, perhaps as a result of the activation of FGF2 or by the angiogenic properties of the fragments of HA; 3) when placed on the chorioallantoic membrane of chicken embryos or injected into nude mice, the resulting xenografts were larger than those formed by control cells (mock transfectants); and 4) immunohistochemical staining revealed increased levels of angiogenesis in the xenografts. The results of this study suggest that membrane-bound hyaluronidase can play an important role in the progression of malignant tumor cells.